TOXICOLOGICAL STUDY OF COPPER OXIDE NANOPARTICLES (CuONPs) AGAINST A TERRESTRIAL GASTROPOD, THE SNAIL *HELIX ASPERSA*: MORPHOMETRIC, BIOCHEMICAL "ANALYSIS" AND HISTOPATHOLOGICAL EFFECTS

BENRADIA, H.[1,2] – MOUMENI, O.[2] – HAMOUDI, F. S.[1] – BERGHICHE, H.[1*]

[1]*Laboratory of Applied Animal Biology, Department of Biology, Faculty of Science, University of Annaba, 23000 Annaba, Algeria*
(e-mail: hamida.benradia@univ-bba.dz; ouissem.moumeni@univ-bba.dz)

[2]*Department of Biology, Faculty of Natural and Life Sciences, University Mohammed El Bachir El Ibrahimi, 43 000 Borj Bou Arreridj, Algeria*
(e-mail: hamoudifatenselma@gmail.com; hindabentoubal@hotmail.ca)

**Corresponding author*
e-mail: hindaberghichebentoubal@gmail.com

(Received 14th Oct 2024; accepted 16th Jan 2025)

Abstract. Chemical contamination of terrestrial ecosystems poses a significant threat as these environments accumulate pollutants from human activities, such as pesticides and heavy metal nanoparticles. Terrestrial gastropods are sentinel organisms considered to be excellent biomonitoring tools for environmental pollution, as they are able to absorb and accumulate chemical substances in their tissues. The aim of the current study was to assess the potential hazards of nanoparticulate copper oxide (NPs-CuO) following chronic exposure on the bioindicator species *Helix aspersa.* The organisms were topically treated for 28 days with concentrations of 1000 and 1500 μg/mL. The effect of NPs-CuO was investigated on morphometric and histopathological alterations and measurements of selected biomarkers. The results showed a physiological disturbances in the weight and shell diameter of treated snails. The compound also induced histopathological changes in the digestive gland of *H. aspersa*. Furthermore, an induction of catalase and GST activities were observed in the hepatopancreas, constituting a cellular defense against the presence of NPs-CuO in the hepatopancreas. These results highlight the potential adverse effects of NPs-CuO on non-target organisms and their utility as reliable bioindicators for assessing nano-ecotoxicological impacts.
Keywords: *nanoparticles, toxicity, NPs-CuO, Helix aspersa, physiological disturbances, biomarkers*

Introduction

Environmental contamination from excessive chemical pollutants produced by industrial and agricultural activities has triggered adverse ecosystem changes, posing serious environmental threats (Rachid et al., 2023). Environmental toxins, including heavy metals and pesticides, pose significant threats to ecosystems by damaging abiotic components (water, air, and soil) and harming biotic communities (plants, animals, and humans) (Bhunia, 2018; Tiwari et al., 2023). Various organs and body systems can be affected by ingestion, inhalation and skin adsorption of these toxic substances. Heavy metals can lead to neurodegenerative disorders, musculoskeletal diseases and reproductive hormonal imbalances, while pesticide exposure can have dangerous effects such as soft tissue sarcomas, ovarian and lung cancers, asthma and endocrine disruption (Alengebawy et al., 2021).

Furthermore, nanotechnology is the development and manufacture of materials with a nanometric dimension less than 100 nm and their application (Du et al., 2019). The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_31873202

use of nanoparticles (NPs) depends on their chemical and physical properties, such as wettability, size and shape, which are important for self-assembly (Dendukuri et al., 2009). In addition, NPs of small size and large surface area enhance reactivity and modify various properties, which a wide range of applications in biological, environmental and medical fields (Trequesser et al., 2014). In agriculture, nanotechnology research and development avenues can facilitate the advanced stages of genetically modified crops, animal feed supplements and new pesticides (Biswal et al., 2012). Recent literature indicates that metal nanoparticles have potential effects against fungal plant pathogens (Rai and Ingle, 2012; Abd-Elsalam, 2013). The antimicrobial activities of certain NPs such as sulfur (S), silver (Ag), copper oxide (CuO), magnesium oxide (MgO) and ZnO have been studied separately or in combination with biopolymers (Rai and Ingle, 2012). Their use is increasing with time, which has caused the so-called chemical pollution of the environment to which the living organisms in the soil are exposed. These nanoparticles present a danger by their nanometric properties which gives them several powers to interfere with the cells and organs of the body. This interaction of this chemical material with organisms could cause a serious threat to human health and agricultural production (Jeevanandam et al., 2018).

Recently, the improvement of environmental pollution diagnostic tools has been promoted in order to cope with pollution problems. Biomonitoring is used as an alternative method for pollution assessment and the implementation of short- and long-term strategies for the protection of environmental resources. It generally depends on the use of living organisms, known as bioindicators, or their responses, known as biomarkers to indicate environmental changes (Li et al., 2010). The terrestrial gastropod snail is considered a bioindicator species widely used by scientists to characterize environmental changes and soil quality, identify or predict damage to ecosystems, and monitor pollution (Dondi et al., 2023). Such snails possess several criteria: their capability to accumulate various xenobiotics in their tissues, their sufficient numbers, wide distribution, easy sampling, their tolerance to metals and organic contaminants, and their significant part in terrestrial food webs (de Vaufleury, 2015; Baroudi et al., 2020;). For these reasons, these animals are increasingly employed as effective sentinel organisms in ecotoxicological monitoring investigations to determine the impact of various pollutants on the quality of the soil ecosystem based on biomarker responses.

The utilization of biochemical biomarkers to evaluate toxicity effects under controlled laboratory conditions remains a valuable approach to provide indications of xenobiotic toxicity (Santana et al., 2022). These include neurotoxic biomarkers such as acetylcholinesterase, a neurotransmitter hydrolase that contributes to the transmission of nerve impulses by the hydrolytic metabolism of acetylcholine into choline and acetate (Bernal-Rey et al., 2020). Glutathione S-transferase biomarker related to oxidative stress, an important enzyme in the conjugation phase, as it combines with contaminants and generates compounds that are more easily excreted (Ribeiro et al., 2022) or malondialdehyde a product of lipid peroxidation, has been widely used as a biomarker of free radical damage in lipid molecules (Demirci-Cekic et al., 2022). Lipid peroxidation is known to cause cellular injury through the inactivation of membrane enzymes and receptors, depolymerization of polysaccharides, and the cross-linking and fragmentation of proteins. Superoxide dismutase, catalase, glutathione peroxidase, glutathione, and glutathione reductase are oxidative stress biomarkers (Li et al., 2020), whereas metallothioneins are widely used

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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as biomarkers of metal contamination by binding and removing toxic metals (Samuel et al., 2021; Ameur et al., 2022).

The mollusk *H. aspersa* (Müller, 1774), (Stylommatophora, Helicidae) has been the subject of intensive studies covering several aspects (Abdel-Azeem and Osman, 2021; Besnaci et al., 2022). The data obtained provides an experimental basis for studying the side effects xenobiotics on non-target organisms. Therefore, the objective of the present study were to assess the potential hazards following a chronic exposure of *H. aspersa* to a nanoparticles copper oxide (CuONPs) according to Hussein et al. (1994) and Radwan et al. (2008). The experiment on growth consisted on measuring body weight and the morphological parameters of shell size (height and width). In addition, the analysis of oxidative stress biomarkers (catalase and glutathione S-transferase) and the histological structure of the snail's digestive gland were determined to provide information on the toxicity of this product.

Material and methods

Experimental design and treatment

The snails *H. aspersa* (Müller, 1774), (Stylommatophora, Helicidae) were collected in April 2020 from a non-contaminated site, Drâa En Naga, El Khroub in Constantine province (36°22'47" N et 6°44'11"E) (*Fig. 1*); a clean site located far from all sources of agricultural, industrial or urban pollution. They were then reared under the controlled conditions described by Gomot (1994) (temperature 20 ± 2°C, photoperiod 18 hL/6 hO, humidity 80 to 90%) and were fed exclusively with lettuce. Mature specimens (body weight 8 ± 0.1 g) were treated topically (100 µl) with two concentrations (1000 and 1500 µg.mL^{-1}) of CuONPs suspended in water and sonificated before the application. A total of 80 snails were used in this experiment. Five replicates of each series of control and treated snails were performed at 0, 7, 14, 21 and 28 days, which can be considered as chronic exposure (Bartlett et al., 2019).

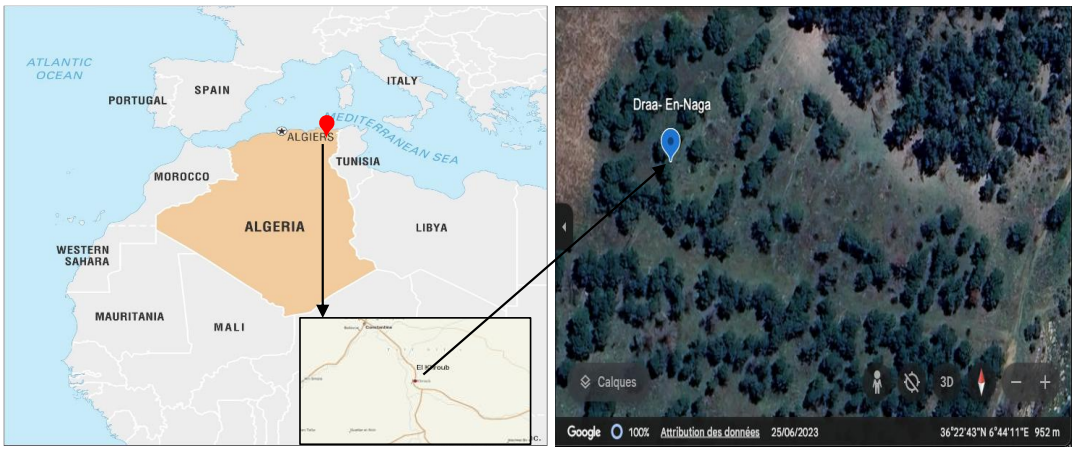

Figure 1. *Sampling site of H. aspersa in Constantine Province, Algeria (El Khroub)*

Morphometric measurements

Three morphometric parameters were recorded on each specimen at different exposure times of 7, 14, 21 and 28 days from the control and treated groups.

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The total weight of the snail was carried out using an Ohauss Type KERN 572 Analytical Balance with a precision of 0.01 g, while the total size of the shell (height and width) was measured using a Tracaeble Fisher scientific caliper.

Determination of biomarkers activity

Catalase (CAT): The activity was determined according to Aebi (1984). The reaction mixture consisted of 0.05 M potassium phosphate pH 7.0 and M H_2O_2 in 0.05 M potassium phosphate working buffer (pH 7.0). One unit of catalase is expressed by the amount of CAT, which decomposes 1 μmol of H_2O_2 per minute at 25°C and pH 7.0 under the specific conditions.

Glutathione S-transferase activity (GST). The glutathione S- transferase activity was analyzed in the snail digestive gland using the method of Habig and Jakoby (1981). Briefly, the digestive gland was individually homogenized in buffer phosphate (0.1 M, pH 6) and centrifuged at 1300 rpm for 30 min, and the supernatant was kept for the enzymatic determination. GST activity was determined by the addition of supernatant to the mixture GSH-CDNB in phosphate buffer (0.1 M, pH 7) using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate. Changes in absorbance were measured every 1 min for 5 min at 340 nm.

In parallel, enzymatic activity was calculated in terms of the protein content of the sample (Bradford, 1976) using Coomassie Bril-liant Blue G250 as a reagent and bovine serum albumin as the standard. Absorbance was measured at 595 nm and reported as μM/min/mg of protein.

Histological studies

For the histological examination, three snails were selected from each experimental group at 21th and 28th day of the experiment, the digestive glands were dissected out and immediately fixed in Bouin's fluid. After 24 h of fixation, specimens were washed and dehydrated in ascending series of ethanol and cleared in xylene and embedded in melted paraplast paraffin wax at 60°C. Serial sections were cut at 5-7 μm and stained with Ehrlich's hematoxylin and counterstained by eosin (Romeis, 1989). Sections were then mounted and covered with glass cover.

Data analysis

Morphometric measurements and enzyme data were analyzed using Graphpads prism graphics (version 9 software). Data were expressed as mean ± SD. Statistical analysis was carried by two-way analysis of variance (ANOVA) followed by Tukey's comparison test with HSD post-hoc analysis was used to assess the differences between the control and treated groups, the concentrations, and durations, with $P < 0.05$, indicating a statistically significant difference.

Results

Effect of copper oxide nanoparticles on morphometric parameters of the H. aspersa snail

The weight

Under normal conditions, the average weight of *H. aspersa* individuals increased significantly on the 21st and 28th days. Treatment of snails with CuONPs at two

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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concentrations (1000 and 1500 μg.mL^{-1}) caused a reduction in the weight of individuals treated at both concentrations (P = 0.0307, P = 0.0008) on the 21st day as compared with control series. This weight reduction continued significantly (P = 0.02, P = 0.0026) during the last week of treatment, after 28 days, compared to the control series at the same time (*Table 1*). It should be noted that snails treated with the CuONPs showed a minimal body weight gain compared with untreated snails. A two-way ANOVA revealed a significant treatment effect ($F_{2, 30} = 6.554$; P = 0.0044), a haily significant time effect ($F_{4, 30} = 35.09$; P = 0.0001), and significant time/treatment interactions ($F_{8, 30} = 2.490$; P = 0.015).

Table 1. *Effect a chronic exposure of snail H. aspersa to a copper oxide nanoparticles (CuONPs) at both concentration 1000 and 1500 μg.mL^{-1} on the ponderal growth (g). (Tukey's test, P < 0.05; x ± SD, n = 5)*

Time	Control	1000	1500
0	9.44 ± 0.15 aA	9.44 ± 0.26 aA	9.64 ± 0.23 aA
7	9.43 ± 0.16 aA	9.66 ± 0.36 aA	9.05 ± 0.54 aA
14	9.85 ± 0.55 aA	9.36 ± 0.75 aA	9.74 ± 1.34 aAB
21	11.38 ± 1.18 aB	10.45 ± 0.12 bAB	10.05 ± 0.61 bB
28	12.97 ± 0.67 aB	11.28 ± 0.01 bB	10.73 ± 0.01 bB

Different capital letters indicate a significant difference between times of the same series; different small letters indicate a significant difference between control and treated series of the same stage (p > 0.05)

The size of shell

Data on the shell width of *H. aspersa* snails from the control and CuO NP-treated series at the two concentrations (1000 and 1500 μg.mL^{-1}) are presented in *Table 2*.

In all series, the evolution of the shells width increased significantly over time up to 28 days. The comparison of the treated series with the control series did not reveal any significant change (P > 0.05) in shell width at the beginning of the treatment, i.e., on days 7 and 14. However, a significant decrease in shell width was observed on day 28 (p = 0.04) with the 1000 μg.mL^{-1} dose and on both 21st (p = 0.0008) and 28th (p = 0.022) days with the higher 1500 μg.mL^{-1} dose. A two-way ANOVA showed a non-significant time effect ($F_{3, 23} = 3.420$; P = 0.034), a significant treatment effect ($F_{2, 23} = 7.169$; P = 0.0037), and significant time/treatment interactions ($F_{6, 23} = 3.173$; P = 0.0204).

Table 2. *Effect a chronic exposure of snail H. aspersa to a copper oxide nanoparticles (CuO NPs) at both concentration 1000 and 1500 μg.mL^{-1} on the shell width (cm). (Tukey's test, P < 0.05; x ± SD, n = 5)*

Time	Control	1000	1500
7	2.77 ± 0.15 aA	2.60 ± 0.26 aA	2.83 ± 0.11 aA
14	2.81 ± 0.62 aA	2.60 ± 0.04 aA	2.60 ± 0.26 aAB
21	2.90 ± 0.13 aB	2.77 ± 0.17 abB	2.57 ± 0.05 bAB
28	2.90 ± 0.15 aB	2.53 ± 0.04 bAB	2.43 ± 0.2 bB

Different capital letters indicate a significant difference between times of the same series; different small letters indicate a significant difference between control and treated series of the same stage (p > 0.05)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The evolution of the shell height of *H. aspersa* snails, controls and treated with copper oxide nanoparticles at the two concentrations (1000 and 1500 μg.mL^{-1}), is presented in *Table 3*.

In both controls and treated snails, shell height increased significantly at day 28. Treatment of *H. aspersa* snails with both concentrations resulted in a significant reduction in shell height on days 21 and 28 of treatment, compared with the control series of the same age. Two-way analysis of variance showed a significant effect of time ($F_{3, 24}$ = 19.22; P = 0.0018), a significant effect of treatment ($F_{2, 24}$ = 16.51; P = 0.0001), and significant time/treatment interactions ($F_{6, 24}$ = 3.984; P = 0.006).

Table 3. *Effect a chronic exposure of snail H. aspersa to a copper oxide nanoparticles (CuO NPs) at both concentration 1000 and 1500 μg.mL^{-1} on the shell height (cm). (Tukey's test, P <0.05; x ± SD, n = 5)*

Time	Control	1000	1500
7	1.54 ± 0.08 aA	1.67± 0.13 aA	1.60 ± 0.31 aA
14	1.80± 0.17 aA	1.71 ± 0.13 aA	1.68 ± 0.13 aA
21	1.90 ± 0.13 aA	1.76 ± 0.06 bA	1.79 ± 0.13 bB
28	2.10 ± 0.06 aB	1.87 ± 0.04 abB	1.82 ± 0.08 bB

Different capital letters indicate a significant difference between times of the same series; different small letters indicate a significant difference between control and treated series of the same stage (p > 0.05)

Effect of copper oxide nanoparticles on enzymatic activities in the snail H. aspersa

Measurements of biomarkers were performed on fragments of the hepatopancreas of *H. aspersa* snails in controls and treated snails with copper oxide nanoparticles at two concentrations (1000 and 1500 μg.mL^{-1}) at 7, 14, 21 and 28 days of exposure. The results obtained for the variation in the enzymatic activities of glutathione S-transferase and catalase were presented in (*Figs. 2* and *3*).

Catalase activity

As shown in *Figure 2*, under normal conditions, specific catalase activity was low and did not vary over time, up to 28 days. Treatment with CuO at both concentrations revealed a highly significant (p < 0.001) induction of catalase activity after 7, 14, 21 and 28 days with a dose-dependent effect as compared to controls (*Fig. 2*). A two-way ANOVA revealed a highly significant treatment effect ($F_{3,23}$ = 79.87; P = 0.0003), a no significant time effect ($F_{2, 23}$ = 186.7; P = 0.0945), and no significant effect time/treatment interactions ($F_{6, 23}$ = 0.882; P = 0.523).

GST activity

In the control series, the enzymatic activity of glutathione S-transferase remained unchanged over time, up to 28 days. On day 7, the data showed that there was no significant (p > 0.05) increase in glutathione S-transferase in the treated series. However, a highly significant dose-dependent effect of glutathione S-transferase was observed on days 14, 21 and 28 with a highly significant (p 0.001) induction (*Fig. 3*). A two-way ANOVA revealed a highly significant time effect ($F_{3, 22}$ = 95.22; P = 0.0001),

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treatment effect ($F_{2,\ 22} = 199.9$; $P = 0.0001$), and time/treatment interactions ($F_{6,\ 22} = 30.32$; $P = 0.0001$).

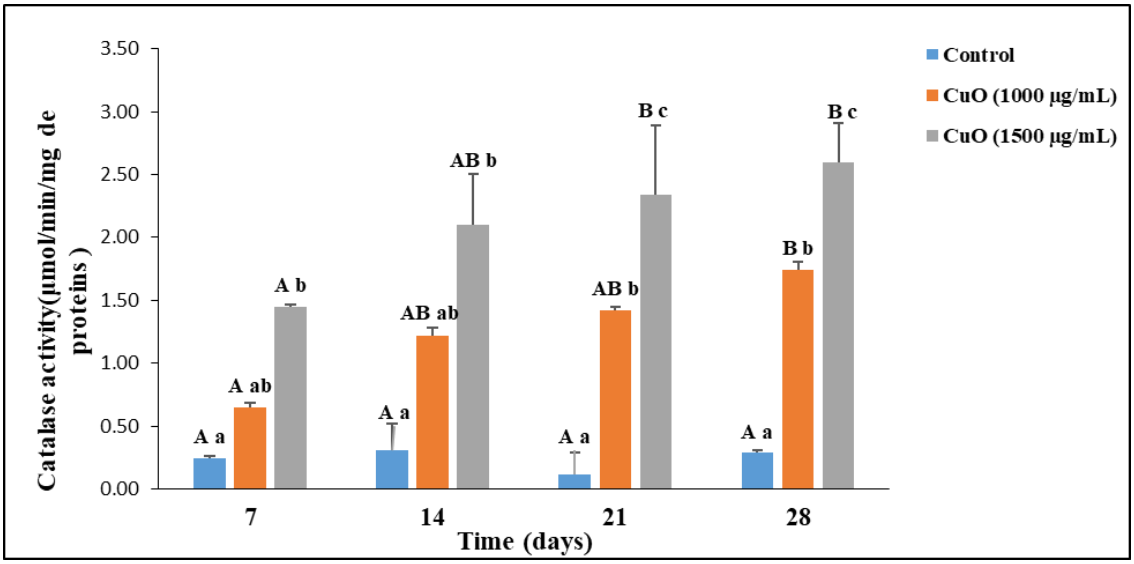

Figure 2. *Effect a chronic exposure of snail H. aspersa to a copper oxide nanoparticles (CuONPs) at both concentration 1000 and 1500 µg.mL^{-1} on specific catalase activity (Error bars represent mean ± SD. Different letters above the bar indicate significant difference at $p < 0.05$). Different capital letters indicate a significant difference between times of the same series; different small letters indicate a significant difference between control and treated series of the same stage ($p > 0.05$)*

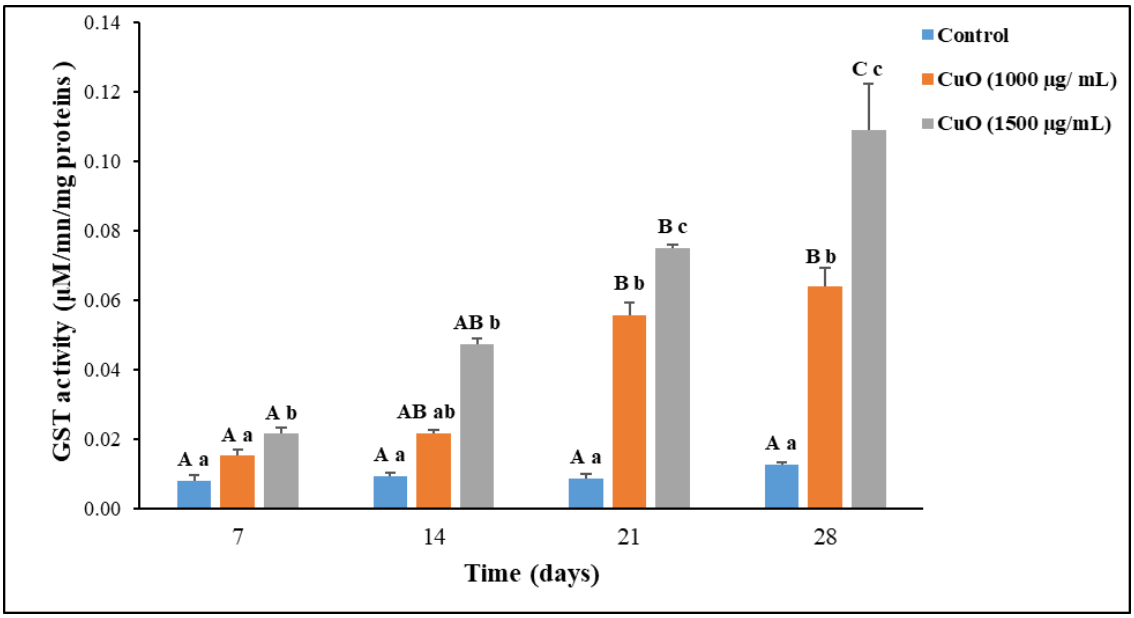

Figure 3. *Effect a chronic exposure of snail H. aspersa to a copper oxide nanoparticles (CuONPs) at both concentration 1000 and 1500 µg.mL^{-1} on glutathione S-transferase activity (Error bars represent mean ± SD. Different letters above the bar indicate significant difference at $p < 0.05$). Different capital letters indicate a significant difference between times of the same series; different small letters indicate a significant difference between control and treated series of the same stage ($p > 0.05$)*

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DOI: http://dx.doi.org/10.15666/aeer/2302_31873202

Effect of copper oxide nanoparticles on the hepatopancreatic tissue of H. aspersa snails

In control snails, the hepatopancreas of snails covers a large part of the visceral mass, and composed of several digestive tubules, each lined with a simple epithelium containing different cell types such as digestive, excretory and calcific cells. They are arranged around a tight lumen (*Fig. 4A*). After a CuONPs exposure at both concentrations 1000 and 1500 µg.mL^{-1} for 28 days, the tissue of the hepatopancreas of snails treated shows a deformation of the acini, an enlargement of the lumen, accompanied a few foci of necrosis after 28 days of treatment in a dose-dependent manner. We also note the presence of necrotic cells accompanied by inflammatory lymphoplasmacytic infiltrates after 28 days of treatment with CuO at 1500 µg.mL^{-1} (*Fig. 4*). These alterations occurred most frequently in snails exposed to the highest concentrations of CuO.

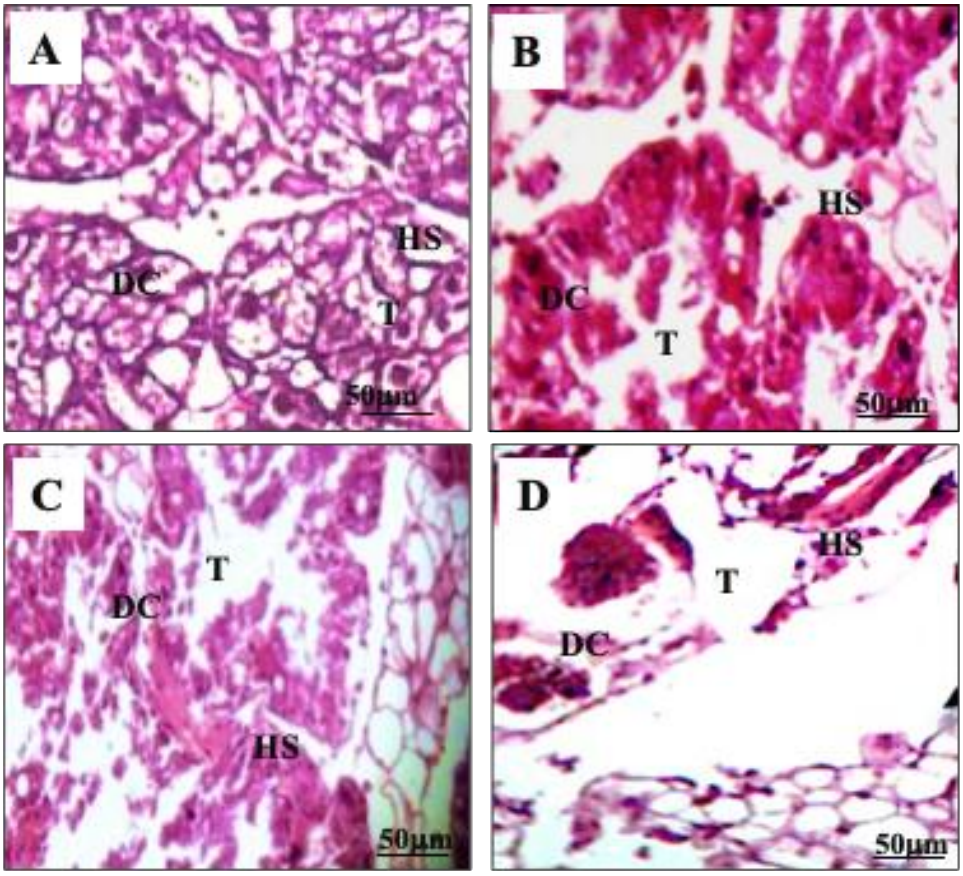

Figure 4. *Histological sections of the hepatopancreas of control snails (A) (normal structure), those treated with different concentrations of CuONPs, 1000 µg.mL^{-1} (28 day) (B) (minimal lesions), 1500 µg.mL^{-1} (C), 1500 µg.mL^{-1} (D) during the 21st, and 28 days, respectively (moderate pathological, and severe pathological change, respectively). Digestive cells (DC), tubulus lumen (T), hemolymphe spaces (HS)*

Discussion

Gastropods are a large group of mollusks, most of which have a rolled or conical shell, which may be extremely small in some species or disappear completely, as in

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slugs (Van Dooren, 2022). Various snail species have been studied, such as *Achatina fulica* (Stylommatophora, Achatinidae) (Cho et al., 2019), *Indothais gradata* (Neogastropoda, Muricidae) (Proum et al., 2016), *H. aspersa* (Stylommatophora, Helicidae) (Abdel-Halim et al., 2013), *Papillifera papillaris* (Stylommatophora, Clausiliidae) (Emilia et al., 2016), Eobania vermiculata (Stylommatophora, Helicidae) (El-Shenawy et al., 2012), *Cantareus apertus* (Stylommatophora, Helicidae) (Mleiki et al., 2016), and *Pomacea canaliculata* (Gasteropodae, Ampullarioidea) (Ramli et al., 2019). These are key species used as bioindicators of soil pollution to assess the toxicity of xenobiotics (El-Gendy et al., 2021). They are considered appropriate sentinel species because different types of pollutants, such as pesticides, metals, nanoparticles and others, tend to accumulate in their digestive glands to allow biomonitoring of pollution (Carbone and Faggio, 2019).

Environmental health has always been threatened by the bioaccumulation of toxic substances in ecosystems, affecting their quality and safety (Okereafor et al., 2020). These contaminants pose a high ecotoxicological risk to terrestrial organisms, particularly for their growth and reproduction (Gomes et al., 2017). This justifies the use of *H. aspersa* as a study model for assessing the health of terrestrial ecosystems with morphometric, biochemical and histopathological approaches. So, the present study attempted to evaluate the impact of soil contamination by CuO-NP. In ecotoxicology, growth assessment is one of the possible indices of physiological integrity (Conti, 2008), where growth rate, however, depends on a series of physiological and/or biochemical processes (Sanders et al., 2021). Furthermore, measurements of length and weight of shell species are used to determine growth patterns. This is also a biological response used as an indicator of how stress affects individuals (Carbone and Faggio, 2019). In this context, our experiment demonstrates that the chronic exposure of *H. aspersa* to copper oxide nanoparticles for 28 days results in a reduction in the total weight of the snails. Concerning the size of the shell of the snails treated by CuO, we observed an inhibition in the evolution of the diameter of the shell compared to the controls throughout the treatment period, which could be explained by an inhibition of the growth hormone as suggested by Gimbert et al. (2006). Our results are similar to those reported by Besnaci et al. (2016), where a decrease in mean weight was observed after exposure of snails *Cantareus aspersus* (Stylommatophora, Helicidae) to food contaminated with iron oxide nanoparticles (Fe_20_3NPs). This weight loss can be explained by the reduction in food consumption observed particularly in treated animals (Shireley et al., 2017). In addition, the growth rate of the snail *Lissachatina fulica* was reduced by the ingestion of dietary nanoplastics for 14 days (Chae and An, 2020). Similar results were obtained by Grara et al. (2015), who treated *H. aspersa* snails by adding increasing concentrations of zinc oxide nanoparticles (ZnO) to the feed (wheat flour), induced a weight decrease. In addition, Bendokhane and Gounache (2013) reported that growth inhibition was represented by a dose-dependent reduction in weight, a reduction in shell diameter and weight, as well as a dose-dependent reduction in food consumption rate and dry weight of waste with behavioral disturbances in *H. aspersa* snails treated with different metal nanoparticles: zinc oxide (ZnO), iron oxide (Fe_2O_3), aluminum oxide (Al_2O_3). In the same context, the work carried out by Schuytema et al. (1994) revealed a reduction in total weight and shell diameter in *C. aspersus* snails exposed to aminocarb, azinphos-methyl, carbaryl, fenitrothion, methyl parathion, paraquat and trichlorfon for 14 days. However, there is no significant impact on the giant African snail *Lissachatina fulica* (Stylommatophora,

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Achatinidae) growth indices (shell diameter and length) after exposure to microplastic fibers for four weeks (Song et al., 2020). Some metals, such as cadmium (Cd), lead (Pb) and Zinc (Zn), have been assessed in various species of terrestrial gastropods (*C. aspersus, H. aspersa, Oxymeris maculate, Lamillidens corrianus*) under laboratory conditions. They were found to have negative effects on shell size, body weight and growth (Kamble and Londhe, 2012).

Several biomarkers are used to characterize and quantify the exposure and harmful effects of various pollutants, which lead to a cascade of biological responses triggered by stress. To analyze the likely negative effects of CuONP on *H. aspersa,* two oxidative stress biomarkers were determined. Catalase is a main component in the antioxidant defense system. It has a role in catalyzing the conversion of hydrogen peroxide to water and oxygen using an elemental cofactor (Chalikani et al., 2004). While the glutathione s-transferase enzyme involves in catalyzing the conjugation of a variety of electrophilic substrates to reduce glutathione and protect the cell against the effects of xenobiotics (Ferrari et al., 2007). In the current study, the catalase and glutathione s-transferase activities in the digestive glands of CuONP-treated *H. aspersa* snails were induced in a dose-dependent manner after 7, 14, 21 and 28 days of exposure. Previous studies have demonstrated that nanomaterials can induce an oxidative stress in animals exposed to them. This significant increase is able for inducing oxidative stress in exposed animals. This significant increase in biomarker activities may be due to increased production of oxygen free radicals, which could stimulate antioxidants to protect cells against damage (Torres et al., 2002). Moreover, an increase in catalase activity has already been observed in *H. aspersa* (Abdel-Halim et al., 2020) and *Lymnaea luteola* (Hygrophila, Lymnaeidae) with ZnONPs (Ali et al., 2012). Regarding engineered nanomaterials (ENMs), numerous publications reported a significant induction of catalase activity. This was observed in *Lucina luteola* (Lucinida, Lucinidae) and in the kidneys and digestive glands of *H. aspersa* treated with titanium dioxide nanoparticles (TiO_2NPs) (Ali et al., 2015; Khene et al., 2017). Thus, the increases in enzyme activity may be attributed to the activation of the natural antioxidant defense system by the mineral. The increased GST suggested that detoxication process versus pro-oxidation forces, mediated by this enzyme, was induced (Elia et al., 2007). This concept was stated by Canesi et al. (1999), where, for example, the increase in GST activity induced by copper treatment may reflect an increased utilization of GSH in conjugation in the metabolism of lipid hydroperoxides and carbonyl compounds formed by the Cu-induced peroxidation of the cellular membrane. In accordance with the present finding, Radwan et al. (2010) and Abdel-Halim et al. (2013, 2020) showed that, GST activity increased in snails; *Theba Pisana* (Stylommatophora, Helicidae) and *H. aspersa* exposed to heavy metal pollution in urban regions. Khene et al. (2017) showed an induced GST activity in the digestive gland of snail *H. aspersa* exposed to TiO_2NPs and in bivalve *Scrobicularia plana,* (Veneroida, Semelidae) exposed to Ag, Au, Cd, and ZnO microparticles (Mouneyrac et al., 2014).

Histopathology is used in biomonitoring programs as a biomarker tool to effectively demonstrate the health status of an organism (Reddy, 2012). It enables in situ assessment of toxic effects in short- and long-term studies (Morales-Caselles et al., 2008). The digestive gland is actively involved in the detoxification and elimination of xenobiotics and is the main site of metabolic processes, particularly metal accumulation and deposition (Canesi et al., 2010). There are few reports on the effects of nanoparticles on the digestive gland of mollusks, therefore, the current research was

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DOI: http://dx.doi.org/10.15666/aeer/2302_31873202

carried out to assess the toxic effects of CuONPs on the structural integrity of the digestive gland of *H. aspersa* experimentally exposed to CuONPs. Our observations of the histological structure of hepatopancreas tissues reveal some alterations occurred most frequently in snails exposed to the highest concentrations 1500 $mg.L^{-1}$ of CuO. This nanoparticle induces a deformation of the acini, an enlargement of the lumen, accompanied a few foci of necrosis, and the presence of necrotic cells accompanied by inflammatory lymphoplasmacytic infiltrates in the tissue of the hepatopancreas of snails in a dose-dependent manner. Based on these findings, the hypothesis is that pollutants were transported by hemocytes to the digestive gland, so exposure to metal induced alterations in its tissue. This is confirmed by Ali et al. (2015), who recorded that excretory cells increased and digestive cells became more flattened in the exposed *Eobania vermiculata* (Müller, 1774) (Stylommatophora, Helicidae) to silver nanoparticles (Ag-NPs) vacuolization in the digestive cells of exposed *Lymnaea stagnalis* (Studer, 1820) (Pulmonata, Lymnaeidae) to thiodan (endosulfan insecticide) was recorded (Sawasdee et al., 2011), which found destruction of tubules, enlarged tubule lumen, vacuoles, and necrosis of the digestive gland of exposed snail *Marisa cornuarietis* (Linnaeus, 1758) (Architaenioglossa, Ampullariidae) to copper (Cu) and lithium (Li). Abd el-atti et al. (2019) found lumen dilatation and vacuolation in the hepatopancreas of crayfish, *Procambarus clarki* (Girard, 1852), (Decapoda, Cambaridae) exposed to CuONPs. According to Osterauer et al. (2010). Necrosis of digestive cells were observed in hepatopancreas in tissues of *M. cornuarietis* treated with Cu and Li. So, the misuse of nanomaterials may be related and reflected to environment and human health. The industrial use of CuO nps must be monitored and regulated. *H. aspersa* is a good bioindicator of nano-ecotoxicological effects. So, the present study confirms that *H. aspersa* snails are sensitive to CuONPs, which represents a major environmental challenge. Thus, the misuse of nanomaterials can be linked to and reflected on the environment and human health.

Conclusion

The industrial use of CuO nanoparticles must to be monitored and regulated. Moreover, *H. aspersa* is a good bioindicator of nano-ecotoxicological effects.

Further, future perspectives and recommendations, the co-occurrence of toxic mixtures, their interactions and their combined toxicity should be studied in detail. We also suggest that more studies be carried out on new approaches to phytoremediation and bioremediation of environmentally toxic substances.

Acknowledgements. This work was supported by the National Fund for Scientific Research to Pr. H. Boudjelida, University of Badji Mokhtar Annaba "Laboratory of Applied Animal Biology", and the Ministry of High Education and Scientific Research of Algeria (University Research-Training Project to Prof. S. Chouahda).

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